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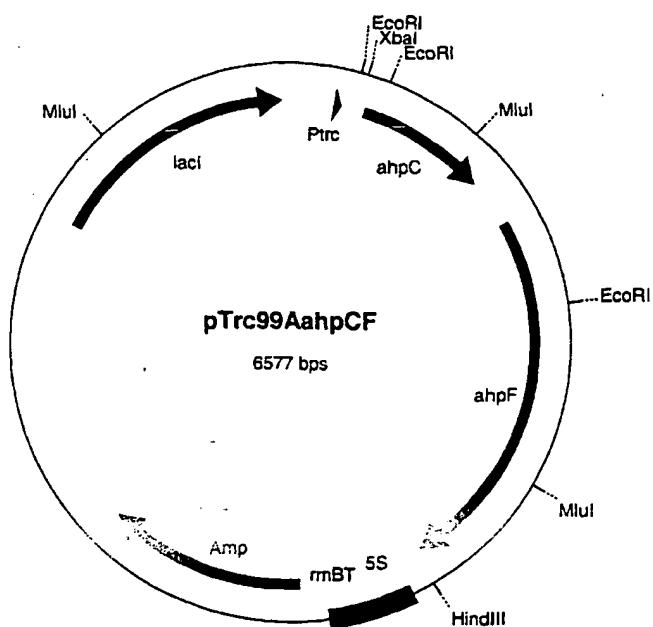
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(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY



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(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.

**Process for the Preparation of L-Amino Acids using Strains of the Enterobacteriaceae Family**

**Field of the Invention**

This invention relates to a process for the preparation of 5 L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which at least one or more of the genes chosen from the group consisting of ahpC and ahpF is (are) enhanced.

**Prior Art**

10 L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known to prepare L-amino acids by fermentation of 15 strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. 20 stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

25 Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for 30 metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine, are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid

5 biosynthesis genes and investigating the effect on the production.

#### Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in

10 particular L-threonine.

#### Summary of the Invention

The invention provides a process for the preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in

15 particularly already produce L-amino acids and in which at least one or more of the nucleotide sequence(s) which code(s) for the ahpC and ahpF genes is (are) enhanced.

#### Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the

20 following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by

30 the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or

protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is

5 in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

10 The process is characterized in that the following steps are carried out:

- a) fermentation of microorganisms of the Enterobacteriaceae family in which one or more of the genes chosen from the group consisting of ahpC and ahpF is (are) enhanced,
- 15 b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and
- c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

20 The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.

Suitable strains, which produce L-threonine in particular, of the genus *Escherichia*, in particular of the species *Escherichia coli*, are, for example

- Escherichia coli TF427
- 5 Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIgenetika MG442
- Escherichia coli VNIIgenetika M1
- Escherichia coli VNIIgenetika 472T23
- 10 Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132.

Suitable L-threonine-producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*, 15 are, for example

- Serratia marcescens* HNr21
- Serratia marcescens* TLr156
- Serratia marcescens* T2000.

Strains from the Enterobacteriaceae family which produce L-20 threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic acid, 25 resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a 30 partial and compensatable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid,

resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine

5 dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement of homoserine kinase, enhancement of threonine synthase,

10 enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form, enhancement of phosphoenol pyruvate synthase, enhancement

15 of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

It has been found that microorganisms of the

20 Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after enhancement, in particular over-expression, of at least one or more of the genes chosen from the group consisting of ahpC and ahpF.

25 The use of endogenous genes is in general preferred. "Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences present in the population of a species.

The nucleotide sequences of the genes of *Escherichia coli*

30 belong to the prior art and can also be found in the genome sequence of *Escherichia coli* published by Blattner et al. (Science 277: 1453-1462 (1997)).

The following information on the ahpC gene and ahpF gene is known, inter alia, from the prior art:

## ahpC gene:

Description: C22 subunit of alkyl hydroperoxide reductase; detoxification of hydroperoxides

EC No.: 1.6.4.-

5 Reference: Ferrante et al.; Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995) Poole and Ellis; Biochemistry 35(1): 56-64 (1996) Nishiyama et al.; Journal of Bacteriology 183(8): 10 2431-2438 (2001)

Accession No.: AE000166

## ahpF gene:

Description: F52a subunit of alkyl hydroperoxide reductase; detoxification of hydroperoxides

15 Reference: Ferrante et al.; Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995) Poole and Ellis; Biochemistry 35(1): 56-64 (1996) Poole et al.; European Journal of Biochemistry 20 267(20): 6126-6133 (2000)

Accession No.: AE000166

The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), the 25 nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

Alleles of at least one or more of the genes chosen from 30 the group consisting of ahpC and ahpF which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used.

To achieve an enhancement, for example, expression of the genes or the catalytic properties of the proteins can be increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the 5 corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is 10 additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the life of the mRNA. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme 15 protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of 20 the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190 25 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80: 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169 30 (1989)), in Hamilton (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

35 Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived

from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80(21): 6557-6561 (1983)) can be  
5 used. A strain transformed with a plasmid vector, wherein the plasmid vector carries at least one or more of the genes chosen from the group consisting of ahpC and ahpF or nucleotide sequences which code for them, can be employed in a process according to the invention.

10 It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al. (Journal of Bacteriology 171: 4617 - 4622 (1989)), conjugation or transduction.

15 It may furthermore be advantageous for the production of L-  
amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate, in  
20 addition to the enhancement of one or more of the genes chosen from the group consisting of ahpC and ahpF.

Thus, for example, one or more of the genes chosen from the group consisting of

- the thrABC operon which codes for aspartate kinase,  
25 homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene which codes for pyruvate carboxylase (DE-A-19 831 609),
- the pps gene which codes for phosphoenol pyruvate  
30 synthase (Molecular and General Genetics 231(2): 332-336 (1992)),

- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- 5 • the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mqo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 10 013 765),
- the thrE gene of *Corynebacterium glutamicum* which codes for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 15 199-209 (1983)),
- the dps gene which codes for the global regulator Dps (Génés & Development 6(12B): 2646-2654 (1992), Accession No. AE000183),
- the hns gene which codes for the DNA-binding protein 20 HLP-II (Molecular and General Genetics 212(2): 199-202 (1988), Accession No. AE000222),
- the lrp gene which codes for the regulator of the leucine Lrp regulon and high-affinity transport systems of branched-chain amino acids (Journal of Biological 25 Chemistry 266(17): 10768-10774 (1991), Accession No. AE000191),
- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994), Accession No. AE000172),

- the fba gene which codes for fructose bisphosphate aldolase (Biochemical Journal 257: 529-534 (1989), Accession No. AE000376),
- the ptsG gene which codes for the glucose-specific IIBC component of the phosphotransferase system PTS (Journal of Biological Chemistry 261(35): 16398-16403 (1986), Accession No. AE000210),
- the ptsH gene of the ptsHICrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- the ptsI gene of the ptsHICrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- the crr gene of the ptsHICrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- the mopB gene which codes for chaperone GroES (Journal of Biological Chemistry 261(26): 12414-12419 (1986), Accession No. AE000487),

can be enhanced, in particular over-expressed.

25 The use of endogenous genes is in general preferred.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of one or more of the genes chosen from the group consisting of ahpC and ahpF, for one or more of the 30 genes chosen from the group consisting of

- the *tdh* gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the *mdh* gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- 5 • the gene product of the open reading frame (orf) *yjfA* (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the gene product of the open reading frame (orf) *ytfP* (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- 10 • the *pckA* gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- the *poxB* gene which codes for pyruvate oxidase (Nucleic Acids Research 14(13): 5449-5460 (1986)),
- 15 • the *aceA* gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- the *dgsA* gene which codes for the *DgsA* regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)) and is also known under the name of the *mlc* gene,
- 20 • the *fruR* gene which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the *cra* gene, and
- 25 • the *rpoS* gene which codes for the  $\sigma^{38}$  factor (WO 01/05939) and is also known under the name of the *katF* gene,

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak 5 promoter or a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

By attenuation measures, the activity or concentration of 10 the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

15 It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of one or more of the genes chosen from the group consisting of *ahpC* and *ahpF*, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing 20 Microorganisms", in: *Overproduction of Microbial Products*, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed 25 batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction 30 to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)] or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994))).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General 5 Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower 10 oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as 15 a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium 20 phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-25 containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be 30 employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

5 Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or

10 10 oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually

15 15 reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958), or it can take place by

20 20 reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine,

25 25 L-homoserine and L-lysine, in particular L-threonine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The minimal (M9) and complete media (LB) for *Escherichia coli* used are described by J.H. Miller (A Short Course in

30 30 Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, ligation, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular Cloning - A Laboratory

Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of *Escherichia coli* is carried out by the method of Chung et al. (Proceedings of the National Academy of Sciences of the United States of 5 America (1989) 86: 2172-2175).

The incubation temperature for the preparation of strains and transformants is 37°C.

Example 1

Construction of the expression plasmid pTrc99AahpCF

10 The *ahpC* and *ahpF* genes from *E. coli* K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequences of the *ahpC* and *ahpF* genes in *E. coli* K12 MG1655 (Accession Number AE000166, Blattner et al. (Science 277: 1453-1462 15 (1997))), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The sequences of the primers are modified such that recognition sites for restriction enzymes are formed. The recognition sequence for *Xba*I is chosen for the *ahpCF1* primer and the recognition sequence 20 for *Hind*III for the *ahpCF2* primer, which are marked by underlining in the nucleotide sequence shown below:

*ahpCF1*: 5' - GCATCTAGACGATAACACGGAGGAAG - 3' (SEQ ID No. 1)

*ahpCF2*: 5' - GCTAAGCTTTGCAGGTGAATC - 3' (SEQ ID No. 2)

25 The chromosomal *E. coli* K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 2400 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and 30 Applications, Academic Press) with *Pfu*-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is cleaved with the restriction enzymes *Xba*I and *Hind*III and

ligated with the vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which has been digested with the enzymes XbaI and HindIII. The *E. coli* strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the 5 ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRI and MluI. The plasmid is called pTrc99AahpCF (figure 1).

10 Example 2

Preparation of L-threonine with the strain MG442/pTrc99AahpCF

The L-threonine-producing *E. coli* strain MG442 is described in the patent specification US-A- 4,278,765 and deposited 15 as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99AahpCF described in example 1 and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar 20 with 50 µg/ml ampicillin. The strains MG442/pTrc99AahpCF and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 1.5 g/l KH<sub>2</sub>PO<sub>4</sub>, 1 g/l NH<sub>4</sub>Cl, 0.1 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g/l 25 glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/l KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 15 g/l 30 CaCO<sub>3</sub>, 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250  $\mu$ l portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the 5 batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with 10 an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) 15 by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AahpCF	5.4	2.6

20 Brief Description of the Figure:

- Figure 1: Map of the plasmid pTrc99AahpCF containing the ahpC and ahpF genes.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- Amp: Ampicillin resistance gene
- 5 • lacI: Gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- ahpC: Coding region of the ahpC gene
- ahpF: Coding region of the ahpF gene
- 10 • 5S: 5S rRNA region
- rrnBT: rRNA terminator region

The abbreviations for the restriction enzymes have the following meaning

- EcoRI: Restriction endonuclease from Escherichia coli  
15 RY13
- HindIII: Restriction endonuclease from Haemophilus influenzae
- MluI: Restriction endonuclease from Micrococcus luteus IFO 12992
- 20 • XbaI: Restriction endonuclease from Xanthomonas campestris

**What is claimed is:**

1. Process for the preparation of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
  - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which one or more of the genes chosen from the group consisting of ahpC and ahpF or the nucleotide sequences which code for these is 10 (are) enhanced, in particular over-expressed,
  - b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its 15 entirety or portions (> 0 to 100%) thereof optionally remaining in the product.
2. Process according to claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are 20 employed.
3. Process according to claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 25 4. Process according to claim 1, wherein the expression of the polynucleotide(s) which code(s) for one or more of the genes chosen from the group consisting of ahpC and ahpF is increased.
5. Process according to claim 1, wherein the regulatory 30 and/or catalytic properties of the polypeptides

(proteins) for which the polynucleotides ahpC and ahpF code are improved or increased.

6. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the 5 Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:

10 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,

6.2 the pyc gene which codes for pyruvate carboxylase,

6.3 the pps gene which codes for phosphoenol pyruvate synthase,

15 6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,

6.5 the pntA and pntB genes which code for transhydrogenase,

20 6.6 the rhtB gene which imparts homoserine resistance,

6.7 the mqo gene which codes for malate:quinone oxidoreductase,

6.8 the rhtC gene which imparts threonine resistance,

25 6.9 the thrE gene which codes for the threonine export protein,

6.10 the gdhA gene which codes for glutamate dehydrogenase,

6.11 the *hns* gene which codes for the DNA-binding protein HLP-II,

6.12 the *lrp* gene which codes for the regulator of the leucine Lrp regulon,

5 6.13 the *pgm* gene which codes for phosphoglucomutase,

6.14 the *fba* gene which codes for fructose bisphosphate aldolase,

10 6.15 the *ptsG* gene which codes for the glucose-specific IIBC component,

6.16 the *ptsH* gene which codes for the phosphohistidine protein hexose phosphotransferase,

15 6.17 the *ptsI* gene which codes for enzyme I of the phosphotransferase system,

6.18 the *crr* gene which codes for the glucose-specific IIA component,

6.19 the *mopB* gene which codes for chaperone GroES,

20 6.20 the *dps* gene which codes for the global regulator Dps,

is or are enhanced, in particular over-expressed, are fermented.

7. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:

7.1 the *tdh* gene which codes for threonine dehydrogenase,

7.2 the *mdh* gene which codes for malate dehydrogenase,

7.3 the gene product of the open reading frame (orf) *yjfA*,

5 7.4 the gene product of the open reading frame (orf) *ytfP*,

7.5 the *pckA* gene which codes for phosphoenol pyruvate carboxykinase,

7.6 the *poxB* gene which codes for pyruvate oxidase,

10 7.7 the *aceA* gene which codes for isocitrate lyase,

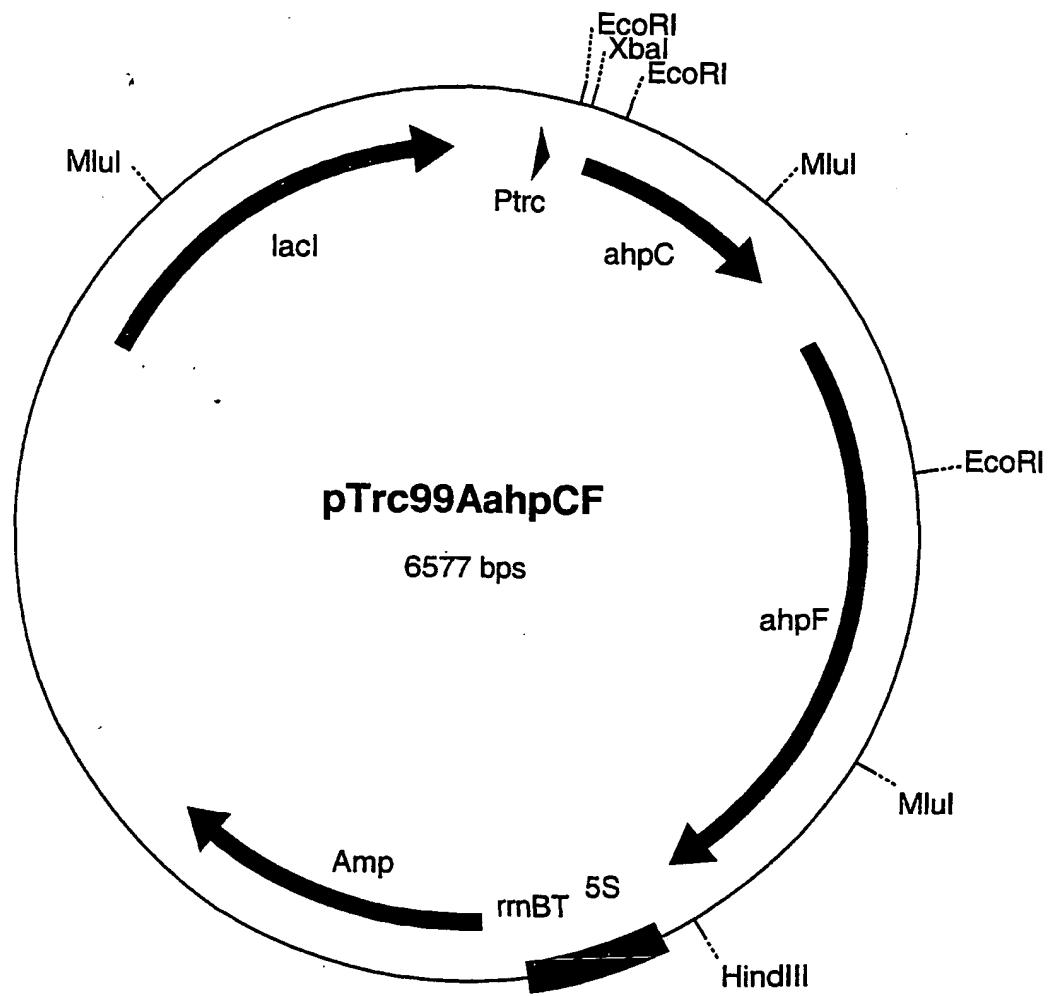
7.8 the *dgsA* gene which codes for the *DgsA* regulator of the phosphotransferase system,

7.9 the *fruR* gene which codes for the fructose repressor,

15 7.10. the *rpoS* gene which codes for the sigma<sup>38</sup> factor

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

Figure 1:



## SEQUENCE PROTOCOL

5 <110> Degussa AG  
<120> Process for the preparation of L-amino acids using  
strains of the Enterobacteriaceae family  
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40

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Declaration under Rule 4.17:  
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Published:  
— with international search report

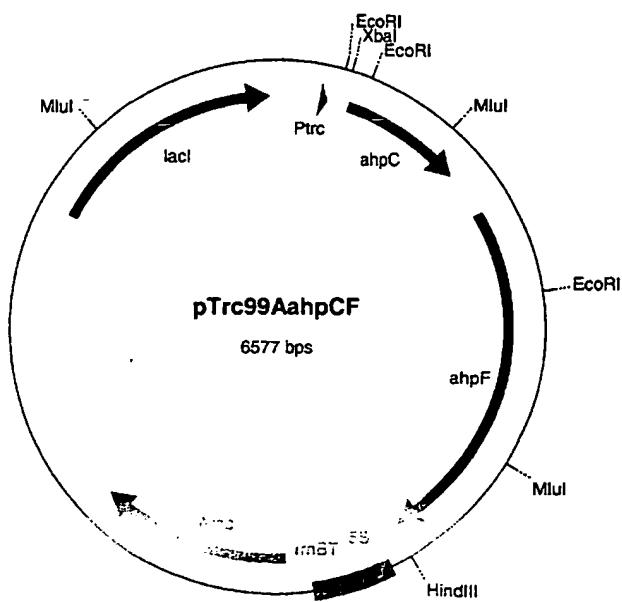
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[Continued on next page]

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY



WO 03/004663 A3



(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.



*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 02/06560A. CLASSIFICATION OF SUBJECT MATTER  
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According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 278 765 A (DEBABOV VLADIMIR G ET AL) 14 July 1981 (1981-07-14) cited in the application the whole document ---	1-7
A	US 5 807 735 A (COYLE CATHERINE L ET AL) 15 September 1998 (1998-09-15) the whole document ---	1-7
A	EP 0 994 190 A (AJINOMOTO KK) 19 April 2000 (2000-04-19) the whole document ---	1-7

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- \*&\* document member of the same patent family

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International Application No  
PCT/EP 02/06560

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAWERS G: "THE ANAEROBIC DEGRADATION OF L-SERINE AND L-THREONINE IN ENTEROBACTERIA: NETWORKS AND PATHWAYS AND REGULATORY SIGNALS" ARCHIVES OF MICROBIOLOGY, BERLIN, DE, vol. 171, no. 1, 1998, pages 1-5, XP002953871 ISSN: 0302-8933 the whole document -----	1-7
E	WO 03 004598 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 & WO 03 004664 A 16 January 2003 (2003-01-16) & WO 03 004665 A & WO 03 004669 A & WO 03 004670 A & WO 03 004671 A & WO 03 004674 A & WO 03 004675 A -----	6
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Information on patent family members

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